Pseudomonas aeruginosa Increases MUC1 Expression in Macrophages Through the TLR4-p38 Pathway

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Running Title: P. aeruginosa increases MUC1 expression in macrophages

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ABSTRACT

Alveolar macrophages (AMs) play a critical role in the clearance of *Pseudomonas aeruginosa* (Pa) from the airways. However, hyper-activation of macrophages can impair bacterial clearance and contribute to morbidity and mortality. MUC1 mucin is a membrane-tethered, high molecular mass glycoprotein expressed on the apical surface of mucosal epithelial cells and some hematopoietic cells, including macrophages, where it counter-regulates inflammation. We recently reported that Pa up-regulates the expression of MUC1 in primary human AMs and THP-1 macrophages, and that increased MUC1 expression in these cells prevents hyper-activation of macrophages that appears to be important for host defense against severe pathology of Pa lung infection. The aims of this study were to elucidate the mechanism by which Pa increases MUC1 expression in macrophages. The results showed that: (a) Pa stimulation of THP-1 macrophages increased MUC1 expression both at transcriptional and protein level in a dose-dependent manner; (b) Both Pa- and LPS-induced MUC1 expression in THP-1 cells were significantly diminished by an inhibitory peptide of TLR4; and (c) LPS-stimulated MUC1 expression was diminished at both the mRNA and protein levels by an inhibitor of the p38 mitogen-activated protein kinase, but not by inhibitors of ERK1/2, JNK, or IKK. We conclude that Pa-stimulated MUC1 expression in THP-1 macrophages is regulated mainly through the TLR4-p38 signaling pathway.
Key words: MUC1, membrane-tethered mucin, macrophage, inflammation, *Pseudomonas aeruginosa*

Nonstandard abbreviations used in this article: aa, amino acid; AM, alveolar macrophage; CF7, control peptide 7; EGFR, epidermal growth factor receptor; ERK1/2, extracellular signal-regulated kinase 1/2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IKK, IκB kinase, IL, interleukin; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MOI, multiplicity of infection; MUC1-CT, MUC1-cytoplasmic tail; NE, neutrophil elastase; NF-κB, nuclear factor-κB; Pa, *Pseudomonas aeruginosa*; PAK, *Pseudomonas aeruginosa* K strain; PMA, phorbol 12-myristate 13-acetate; sTNFR1, soluble tumor necrosis factor-α receptor 1; TLR, Toll-like receptor; TNF, tumor necrosis factor; qRT-PCR, quantitative reverse transcriptase-polymerase chain reaction
INTRODUCTION

*Pseudomonas aeruginosa* (Pa) a Gram-negative opportunistic pathogen that causes nosocomial infection and community acquired infection in at risk host such as patients with chronic airway diseases including chronic obstructive pulmonary disease (COPD) and cystic fibrosis \(^{[1-3]}\). Pa lung infection is encountered by alveolar macrophages (AMs), which sense pathogen-associated molecular patterns through pattern recognition receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs), culminating in neutrophil recruitment to the airways and bacterial elimination \(^{[4-7]}\). Although inflammatory response is essential to the airway defense against Pa infection \(^{[8-10]}\), uncontrolled inflammation caused by AMs in the absence of counter-regulatory mechanisms may be detrimental, contributing to bystander damage to host tissues \(^{[11, 12]}\). Therefore, understanding the mechanisms regulating airway inflammatory responses and resolution of inflammation are highly important.

MUC1 (MUC1 in humans and Muc1 in animals) mucin is a type 1 membrane-spanning glycoprotein expressed on the surface of epithelial cells and some hematopoietic cells \(^{[13]}\). Muc1 deficient mice exhibited hyper-inflammation and enhanced bacterial clearance in a Pa pneumonia model \(^{[14]}\). Subsequent studies demonstrated that up-regulation of Muc1 in the Pa infected lung was associated with diminished levels of proinflammatory mediators (e.g. tumor necrosis factor [TNF], IL-8) and reduced lung injury *in vivo* \(^{[15, 16]}\) suggesting an important anti-inflammatory role of Muc1 during the resolution phase of Pa infection. Our previous *in vitro* studies revealed that the paracrine TNF-TNFR1-ERK1/2-Sp1 signaling pathway is responsible for MUC1 up-regulation in lung epithelial cells \(^{[17-20]}\). We recently reported that MUC1 expression by both
human and mouse AMs was substantially up-regulated in Pa-stimulated or classically activated (M1) macrophages compared with unactivated (M0) and alternatively activated (M2) macrophages [21]. Further, MUC1 expression by macrophages was associated with attenuated innate immune responses such as phagocytic activity and cytokine production [21]. These results support a growing body of evidence suggesting a pivotal, immunomodulatory function of MUC1 in a subpopulation of macrophages during Pa lung infection. However, the underlying molecular mechanisms regulating MUC1 expression by macrophages are unknown.

Here, we report that Pa-induced MUC1 upregulation in macrophages involves activation of TLR4-p38 mitogen-activated protein kinase (MAPK) but, unlike airway epithelial cells (AEC), does not require stimulation by TNF. Since MUC1 in macrophages has been shown to control the production of TNF [5] as well as other inflammatory mediators, the present results support the immunomodulatory role of MUC1 in macrophages during Pa infection by preventing overexpression of TNF and other inflammatory mediators through upregulation of MUC1 [15, 21]. Thus, timely upregulation of MUC1 in macrophages during Pa infection seems to be crucial to regulate inflammation in the acute phase of Pa infection and circumvent severe pathology of Pa-associated pneumonia.

MATERIALS AND METHODS

Cell Culture. Human monocyte THP-1 cells (ATCC, Manassas, VA) were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1.0 mM sodium pyruvate and incubated at 37°C and 5% CO2. The cells were reseeded at 2.5 x 10⁶
cells/well in 6-well culture plates and treated with 200 nM of phorbol 12-myristate 13-acetate (PMA) for 48 hours to induce macrophage differentiation as described [21].

**Stimulation of THP-1 Macrophages with Pa, LPS, or TNF.** Pa strain K (PAK) is a nonmucoid, piliated, and motile strain (kindly provided by Dr. Alice Prince, Department of Pediatrics, Columbia University, New York, NY) and was used as Pa throughout this project. Bacteria were cultured overnight in Luria-Bertani broth supplemented with 60 μg/ml carbenicillin, washed with PBS, and concentrations determined spectrophotometrically using an OD$_{600}$ of 0.5 = $5.0 \times 10^8$ colony forming units/ml [22]. All the Pa strains were heat-killed at 60°C for 30 min [21] prior to treatment of the cells. PMA-differentiated THP-1 macrophages were unstimulated or stimulated for 24 hours with increasing multiplicities of infection (MOIs) of heat-killed Pa, increasing concentrations of Pa LPS (Sigma) as described [21]. In some experiments, the THP-1 cells were pretreated for 1 hour with 10 μM of inhibitors for p38 (SB202190) [23], c-Jun N-terminal kinases (JNK) (SP600125), extracellular signal-regulated kinases 1/2 (ERK1/2) (U0126) [17, 19], 5 μM BAY11-7082 nuclear factor-κB (NF-κB) [24] (all from Santa Cruz Biotechnology, Santa Cruz, CA), or DMSO vehicle control as previously described [25], or with 5.0 μM of the TLR4 inhibitory VIPER peptide or its inactive control peptide 7 (CP7) (Novus Biologicals, Littleton, CO) as previously described [26].

**MUC1 Immunoblotting.** The mature MUC1 protein consists of two non-covalently associated polypeptide subunits, an NH$_2$-terminal extracellular MUC1-α subunit (>250 kDa) and a COOH-terminal MUC1-β subunit (25kDa) containing an evolutionally conserved cytoplasmic tail (MUC1-CT), that are dissociated in SDS-PAGE. In this study, we used anti-MUC1-CT (CT2)
hasmter monoclonal antibody to quantitate MUC1-β subunit protein expression level by Western blot. Briefly, cells were lysed with PBS (pH 7.2) containing 1.0% Triton X-100, 1.0% sodium deoxycholate, and 1.0% protease inhibitor cocktail (Sigma, St. Louis, MO) as described [21]. Equal protein aliquots were resolved by SDS-PAGE and subjected to immunoblot analysis using CT-2 antibody (kindly provided by Dr. Sandra Gendler, Mayo Clinic College of Medicine, Scottsdale, AZ). Immunoblotting for MUC1-β with the CT2 antibody typically produces 2 or 3 immunoreactive bands with an upper 20-25 kD band corresponding to the MUC1-β protein with a single N-linked carbohydrate at the asparagine-36 residue and a lower 15-17 kD band corresponding to the unglycosylated isoform [27]. To control for protein loading and transfer, the blots were stripped and reprobed for β-actin.

**MUC1 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR).** Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and converted to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Pretreatment with TaqMan Universal Master Mix II and the AmpErase Uracil N-Glycosylase Kit (Applied Biosystems) was performed to minimize carryover contamination, after which the cDNA was amplified using the StepOnePlus Real-Time PCR system (Applied Biosystems) using primers for MUC1 (Assay ID, Hs00159357) (Life Technologies, Carlsbad, CA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Assay ID, Hs02758991) as we previously described [21].

**Quantification of TNF Levels by ELISA.** Culture supernatants from THP-1 macrophages stimulated with Pa or LPS were processed for TNF ELISA according to the manufacture’s protocol (R&D Systems) as we previously described [21].
**Statistical Analysis.** Values for MUC1 mRNA and TNF levels were expressed as means ± SEM. A two-tailed Student t-test was used for comparison between two groups and analysis of variance (ANOVA) for more than two groups. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

Pa stimulation increases MUC1 expression by macrophages in a TLR4-dependent manner.

To study molecular mechanisms regulating MUC1 protein expression in Pa-stimulated macrophages, we employed a human monocyte cell line THP-1 that recapitulate MUC1 expression pattern of human alveolar macrophages as well as human blood monocyte-derived macrophages \(^{[21]}\). As shown in figure 1A, MUC1 expression level in unstimulated THP-1 cells was relatively low, whereas stimulation with an increasing dose of Pa for 24 hours increased MUC1 protein levels in a dose-dependent manner. Since it has been shown that macrophages recognize Pa through TLR4 \(^{[5]}\), we examined whether Pa-induced MUC1 up-regulation in macrophages also involves TLR4. Stimulation of the THP-1 cells with Pa increased MUC1 protein levels by 5.4 fold over basal level, whereas pretreatment of THP-1 cells with the viral inhibitory peptide of TLR4 (VIPER) reduced Pa-induced MUC1 RNA levels down to 1.3 fold (Figure 1 C&D). In contrast, control peptide CP7 did not affect Pa-induced MUC1 expression in THP-1 cells. These suggest that Pa-induced MUC1 expression in THP-1 cells is predominantly mediated through TLR4 activation.
LPS increases MUC1 expression in macrophages through the TLR4-p38 Signaling Pathway. Next, we investigated whether or not activation of TLR4 by LPS increases MUC1 level in THP-1 cells. As shown in figure 2A, treatment of THP-1 cells with increasing doses of LPS for 24 hours increased MUC1 protein level (Figures 2A, 2B). Treatment with LPS for 4 hours also increased RNA level of MUC1 by 4 fold over basal level in THP-1 cells, whereas pretreatment with VIPER decreased LPS-induced MUC1 RNA level to 1.5 fold (Figure 2C). To validate VIPER for its inhibitory efficacy for TLR4, LPS-induced TNF levels in culture supernatant were quantitated by TNF ELISA, which showed almost a complete inhibition of LPS-induced TNF by VIPER but not by CP7 control peptide (Figure 2D). To further understand which signaling pathway(s) downstream of TLR4 is involved in MUC1 expression by THP-1 cells, we investigated the effect of pharmacological inhibitors for p38, p42/44 ERK MAPKs, JNK and IKK. Stimulation of THP-1 cells with LPS in the presence of the p38 inhibitor (SB202190) \(^{[23]}\) decreased MUC1 RNA level compared with LPS alone or LPS plus the DMSO vehicle control (Figures 3A). By contrast, stimulation of the cells with LPS in the presence of the JNK inhibitor (SP600125), the ERK inhibitor (U0126) \(^{[17, 19]}\) and the synthetic IKK inhibitor (Bay 11-7082) \(^{[24]}\) had no effect on MUC1 RNA levels (Figure 3B). Finally, stimulation of THP-1 cells with LPS plus the p38 inhibitor (SB202190) also decreased MUC1 protein levels compared with LPS alone (Figure 3C&D). In conclusion, our collective results indicate that Pa/LPS induced MUC1 up-regulation in THP-1 cells at both the gene and protein levels occur through the TLR4-p38 pathway.

DISCUSSION
Inhaled bacterial pathogens are entrapped by mucus overlaying airway epithelial cells and expelled by mucociliary clearance [28]. Failure of the normal clearance mechanisms often leads to bacterial infection, initiating a cellular innate immune response. Although inflammation are essential to the airway defense against Pa infection, excessive inflammation caused by AMs is accountable for severe pathology of Pa-associated pneumonia [11, 12]. Recent findings showed that the immunomodulatory function of MUC1 plays important role in the fine tuned balance of innate immune response by attenuating activation of innate immune receptors, cytokine production and phagocytic activity [14-16, 21, 29-34]. In the present study, we investigated the underlying molecular mechanisms regulating MUC1 expression during Pa infection using THP-1 cells that preserve the characteristics of MUC1 expression as seen in human AMs as well as human monocyte-derived macrophages [21]. Our results showed that Pa stimulation of THP-1 macrophages increased MUC1 gene transcription and protein expression level through activation of TLR4 (Figure 1&2) and the p38 mitogen-activated protein kinase signaling pathway (Figure 3). These results indicate that activation of TLR4 by Pa concomitantly is sufficient induces innate immune responses [4, 5] and MUC1 up-regulation, whereas increased MUC1 counter regulates Pa-stimulated immune responses in macrophages [15, 21].

In a mouse model of Pa lung infection, production of TNF following Pa lung infection was accountable for about 70% of Pa-induced MUC1 up-regulation in lung [15]. Recent in vitro studies revealed differential regulation of MUC1 in AEC and AM. It has been shown that MUC1 is spontaneously expressed in airway epithelial cells, whereas autocrine TNF but not Pa is the major stimulant for MUC1 up-regulation [17, 19, 20]. Further, others and we recently reported that macrophages but not AEC are the major source of TNF in Pa-infected lungs [5, 20]. Although Pa or
LPS stimulation increased TNF release from THP-1 cells, treatment of THP-1 cells with TNF failed to induce MUC1 up-regulation (data not shown). Therefore, in contrast to its regulation in AEC, our current study demonstrated that Pa-activated TLR4 but not autocrine TNF is directly responsible for MUC1 up-regulation in macrophages (Figure 1&2). The results of our present study suggest that mechanisms involved in the regulation of MUC1 in macrophages and AEC are different, the former relying on direct TLR4 activation while the latter requiring TNF in a paracrine manner.

Since MUC1 suppresses LPS-induced NF-κB activation in macrophages \[^{[29]}\] and NF-κB activation following LPS priming of macrophages allows pro-IL-1β production \[^{[35]}\], it is possible to speculate that MUC1 might also suppress inflammasome activation during Pa lung infection as recently suggested (Ng, Menheniott et al. 2016; Ng, Sutton 2016). Since the current study suggests that Muc1 is an inducible protein in macrophages, further investigation is necessary to clarify whether up-regulated Muc1 in macrophages might suppress inflammasome-associated lung pathology during Pa pneumonia. Nevertheless, these collective findings emphasize the importance of MUC1 as a negative regulator of macrophage-generated inflammatory responses. Additional studies are currently underway to identify subpopulations of macrophages that express MUC1 in acute phase of Pa lung infection, as well as to consolidate the immunomodulatory function of MUC1 in macrophages in the context of the pathology of Pa lung infection. In summary, the current studies demonstrate that Pa stimulates MUC1 expression in macrophages through the TLR4-p38 pathway and also increases TNF production, which in turn, augments MUC1 expression in airway epithelial cells suggesting an immunomodulatory role of MUC1 during Pa infection.
DISCLOSURE

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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REFERENCES


FIGURE LEGENDS

Figure 1. Pa induces MUC1 expression by THP-1 macrophages in a dose-dependent manner. (A, C) Confluent PMA-differentiated THP-1 macrophages were untreated or pretreated for 1 hour with the VIPER peptide or CP7 control peptide prior to treatment for 24 hours without or with heat-killed Pa at the indicated MOIs or MOI=10. Equal protein aliquots of cell lysates were processed for MUC1-β immunoblotting. To control for loading and transfer, the blots were
stripped and reprobed for β-actin. (B, D) Densitometric analysis of the blots in (A, C). Data are means ± SEM of combined results from 3 separate experiments. **, significantly different at $P < 0.05$. #, not significantly different at $P > 0.05$.

**Figure 2.** LPS induces MUC1 expression by THP-1 macrophages through TLR4. (A) Confluent THP-1 macrophages were unstimulated or stimulated for 24 hour with increasing concentrations of LPS as indicated. Equal protein aliquots of cell lysates were processed for MUC1-β immunoblotting. (B) Densitometric analysis of the blots in (A). Data are means ± SEM of combined results from 3 separate experiments. (C, D) Confluent THP-1 macrophages were untreated or pretreated for 1 hour with the VIPER peptide or CP7 control peptide prior to stimulation for 4 hour with LPS (100 ng/mL) and the cell lysates and supernatant were subjected to quantitation of MUC1 mRNA levels by qRT-PCR and TNF levels by ELISA respectively. MUC1 mRNA level normalized to the GAPDH mRNA level (n =3). Data are means ± SEM of combined results from 3 separate experiments. **, significantly different at $P < 0.05$. #, not significantly different at $P > 0.05$.

**Figure 3.** LPS induces MUC1 expression by THP-1 macrophages through p38. Confluent THP-1 macrophages were untreated or pretreated for 1 hour with inhibitors of (A) p38 (SB202190), (B) JNK (SP600125), ERK1/2 (U0126), IKK (BAY11-7082) or DMSO vehicle control. The cells were unstimulated (Control) or stimulated for 24 hours with LPS (100 ng/mL). The cell lysates were subjected to quantitation of MUC1 mRNA levels by qRT-PCR. MUC1 mRNA level normalized to the GAPDH mRNA level (n =3). (C) Equal protein aliquots of cell lysates were processed for MUC1-β immunoblotting. To control for loading and transfer, the blot was stripped
and reprobed for β-actin. (D) Densitometric analysis of the blot in (C). Data are means ± SEM of combined results from 3-5 separate experiments. **, significantly different at $P < 0.05$. #, not significantly different at $P > 0.05$. #, not significantly different at $P > 0.05$. 
Figure 1. Pa increases MUC1 in a dose-dependent manner through the activation of TLR4 in macrophages.
Figure 2. LPS-induced TLR4 activation increases MUC1 up-regulation in dose-dependent manner in macrophages.

**Figure Details**

**A**
- LPS (ng/mL) table: 0, 0.1, 1, 10, 100
- Western blot for MUC1 and β-actin
  - MUC1-β at 25 kd and 42 kd

**B**
- MUC1/β-actin ratio graph
  - LPS (ng/mL) range: 0.1, 1, 10, 100
- Bar graph showing up-regulation

**C**
- MUC1 gene fold increase graph
  - Control, LPS, LPS+CP7, LPS+Viper

**D**
- TNF (ng/mL) graph
  - Control, LPS, LPS+CP7, LPS+Viper
  - Symbols: # (p < 0.05), ** (p < 0.01)
Figure 3. LPS-induced MUC1 up-regulation is mediated through the activation of TLR4-p38 MAPK signaling pathway

(A) MUC1 gene (fold change) in different groups: Control, LPS, p38+LPS, and DMSO+LPS.

(B) MUC1 gene (fold increase) in different groups: Con, LPS, ERK+LPS, JNK+LPS, NFκB+LPS.

(C) Table showing the effect of LPS, p38, and DMSO on MUC1 expression:

<table>
<thead>
<tr>
<th>Condition</th>
<th>LPS</th>
<th>p38</th>
<th>DMSO</th>
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<tr>
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(D) MUC1/β-actin expression in different groups: Control, LPS, p38+LPS, and DMSO.